

Role of Cathelicidin Peptides in Bovine Host Defense and Healing

Linda Tomasinsig · Monica Benincasa · Marco Scocchi ·
Barbara Skerlavaj · Alessandro Tossi · Margherita Zanetti ·
Renato Gennaro

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Abstract The *in vitro* antimicrobial activities and biological effects on host cells were compared for the bovine cathelicidins BMAP-28, an alpha-helical AMP, and Bac5 and Bac7, proline-rich AMPs. Our results confirm that the broad-spectrum activity of BMAP-28 correlates with a high capacity to interact with and permeabilize bacterial membranes, whereas the proline-rich AMPs selectively internalize into the cytoplasm of susceptible Gram-negative bacteria with a non-lytic mechanism. All peptides efficiently translocated into mammalian fibroblastic cells, but while Bac5 and Bac7(1–35) localized to nuclear structures and induced cellular proliferation, BMAP-28 associated with mitochondria and did not induce proliferation. Moreover, BMAP-28 was considerably more cytotoxic than the proline-rich peptides due to cytolytic and pro-apoptotic effects. Our results highlight important functional differences among the bovine cathelicidins and suggest that they contribute to an integrated response of the host to infection, with distinct but complementary activities.

Keywords Antimicrobial peptide · Cathelicidin · Alpha-helical · Proline-rich · Membranolytic

Introduction

Antimicrobial peptides (AMPs) are evolutionarily ancient and integral components of the antimicrobial arsenal in virtually every organism, playing an important role in the host defense [11, 35]. Numerous different families of AMPs have been identified in plants and animals, each comprising distinct sets of evolutionarily related peptides with characteristic structural features [29]. A common and distinctive element of all these peptides is the ability to exert a direct antimicrobial activity *in vitro*, on a wide spectrum of microbial pathogens.

In mammals, the best known AMP gene families are the cathelicidins [32, 34] and defensins [21], which, in addition to providing a first line of defense against invading microbes, also afford protection against infection by acting as alarmins [31], stimulating other innate or adaptive immune responses [9, 15] and/or promoting wound healing [12]. These peptides can be expressed by both phagocytic and epithelial cells and can therefore act rapidly in tissues at the interface with the external environment, which are most exposed to the microbial biota and infection by pathogens. The tissue distribution and size of these families can, however, differ significantly among mammals. For instance, while up to a dozen distinct cathelicidin AMPs have been described in pigs, only one cathelicidin seems to be present in many other mammals, including rodents, carnivores and primates [33, 36]. Furthermore, while the single human cathelicidin is widely expressed in both myeloid and epithelial cells, bovid cathelicidins seem to be exclusively of myeloid origin, with no published evidence of expression in epithelial cells [25, 32].

Cathelicidins are characterized by a well conserved, cathelin-like, N-terminal pro-peptide, from which the C-terminal AMPs are proteolytically released on myeloid

L. Tomasinsig · B. Skerlavaj · M. Zanetti
Department of Biomedical Science and Biotechnology,
University of Udine, Udine, Italy

M. Benincasa · M. Scocchi · A. Tossi (✉) · R. Gennaro
Department of Life Sciences, University of Trieste, Trieste, Italy
e-mail: atossi@units.it

cell degranulation or secretion from epithelial cells [32]. A striking feature of artiodactyl cathelicidins is their structural heterogeneity. Bovine BMAP-27, -28 and -34 display the classical amphipathic α -helical structure also found in many amphibian and insect AMPs; ‘dodecapeptide’ has a small β -hairpin structure reminiscent of some insect and crustacean AMPs; the linear proline-rich Bac5 and Bac7 show some analogies to insect proline-rich AMPs; indolicidin displays a peculiar tryptophan-rich structure [24]. This structural diversity reflects in a functional diversity, so that for example the helical BMAPs are thought to kill bacteria principally by membrane lysis [2, 17, 22], while proline-rich AMPs make use of a specific transport system to internalize into Gram-negative bacterial cells and hit internal targets [13, 19]. This functional diversity also extends to a differential cytotoxicity toward host-cells at higher concentrations, and more importantly reflects on non-toxic effects of these AMPs on host cells at lower concentration, related to warding off infection or promoting healing [8, 12, 26].

In this paper, we compare the *in vitro* antimicrobial, cytotoxic and host-cell modulating activities of the α -helical BMAP-28 and proline-rich Bac5 and Bac7(1–35), the N-terminal part of Bac7, the minimum sequence retaining the activity of the natural peptide, underlining their different modes of action. An increased understanding of these activities is desirable as the cathelicidins likely act as endogenous antibiotics, helping to defend cattle from economically deleterious diseases such as mastitis, and might represent lead compounds for novel antibiotics to be used in the livestock and dairy industries.

Materials and Methods

Peptides

Peptide syntheses were carried out using the Fmoc chemistry, as described previously [2, 17, 22], and were verified using ES-MS spectrometry (Applied Biosystems/MDS Sciex or Bruker Esquire 4000 controlled by the Esquire Control software v.5.3). Peptides were dissolved in a water/acetonitrile (1/1), 0.1% (v/v) formic acid solution and ionized by ESI in positive ion polarity. Main ESI and MS acquisition parameters were the following: ESI source: 4,000 V; mass range (m/z): 500–1,500, Max accumulation time: 200 ms, ICC target: 50,000, scan rate: 13,000 m/z/s. Ion trap voltages were automatically controlled with the Smart Parameter Setting Options setting a target mass of 800 m/z, a compound stability of 50%, and a Trap Drive level of 100%.

Concentrations of stock solutions, prepared with accurately weighed peptides, were verified spectrophotometrically [30]. Fluorescein or BODIPY (Invitrogen, CA)

labeling of peptides was conducted as described previously [5, 19, 26].

Antimicrobial Activity Assays

The antibacterial and antifungal activity of the peptides was tested on clinical isolates or collection strains. All the bacterial strains were stored at $-80\text{ }^{\circ}\text{C}$ and routinely grown onto Mueller–Hinton (MH, Becton–Dickinson, Sparks, MD, USA) agar plates. Fungi were grown onto Sabouraud agar plates at $30\text{ }^{\circ}\text{C}$ for 48 h; the inoculum suspensions were prepared by picking five colonies and suspending them in 5 ml of sterile PBS. The minimum inhibitory concentration values (MICs) were determined by the broth microdilution susceptibility test following the guidelines of the NCCLS with mid-log phase cultures. Serial two-fold dilutions of each peptide were prepared (final volume of 50 μl) in 96-well polypropylene microtiter plates with MH broth for bacteria and RPMI-1640 for fungi. Each dilution series included control wells without peptide. A total of 50 μl of the adjusted inoculum (approximately 5×10^5 cells/ml for bacteria or 5×10^4 cells/ml for fungi, in the appropriate medium) was added to each well. To evaluate the MIC, microtiter plates with bacteria were incubated at $37\text{ }^{\circ}\text{C}$ overnight, while those with fungi were incubated at $30\text{ }^{\circ}\text{C}$ for 48 h.

Bacterial Cell Permeabilization and Internalization Studies

Propidium iodide (PI) uptake determinations were carried out with the *E. coli* HB101 and ATCC 25922, and *S. aureus* ATCC 25923 strains, using a Cytomics FC 500 flow-cytometry instrument (Beckman-Coulter, Inc., Fullerton, CA) equipped with an argon laser (488 nm, 5 mW) and photomultiplier tube fluorescence detectors for filtered light (610 nm for PI detection and 525 nm for BODIPY and FITC detection). Bacterial uptake of labeled AMPs was also determined by flow cytometry as described previously [5]. Briefly, 1×10^6 CFU/ml mid-log phase bacteria were harvested and incubated in MH broth with the labeled peptides at $37\text{ }^{\circ}\text{C}$ for 10 min. Treated cells were analyzed directly without washing or after a four times wash with buffered high-salt solution. The quenching effect of trypan blue (TB) was evaluated after incubation of the peptide-treated cells with 1 mg/ml of the dye at room temperature for 10 min. At least 10,000 events were acquired for each sample and data analysis performed with the FCS Express3 software (De Novo Software, Los Angeles, CA). Data are expressed as average MFI (mean fluorescence intensity) \pm S.D.

For confocal laser scanning microscopy (CLSM), the same bacterial cells as used for flow-cytometric studies were exposed to labeled peptide for 10 min, washed four

times with buffered high-salt solution and 10 μl of each bacterial suspension was placed onto a slide and covered with a cover-glass to obtain an unmovable layer of cells. Bacterial cells were examined without any fixation protocol with a Nikon C1-SI confocal microscope, using an oil immersion objective lens. The optimum photomultiplier setting was determined in preliminary experiments performed ad hoc, and then the same setting was used for all samples. The image stacks collected by CLSM were analyzed with EZ-C1 FreeViewer software (Nikon Corporation) and ImageJ 1.40 g software (Wayne Rasband, National Institutes of Health, USA).

Cell Culture and Confocal Laser Microscopy

NIH 3T3 murine fibroblastic cells were cultured under 5% CO_2 at 37 °C as exponentially growing subconfluent monolayers in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS) and 2 mM glutamine. For confocal microscopy analysis, cells were seeded on glass bottom dishes (Willco Wells BV) and incubated overnight at 37 °C in complete DMEM. The cell culture medium was then replaced with DMEM containing 2% FCS and 1.5 μM each fluorescein-labeled peptide and incubation continued for 15 min. After extensive washings, cells were examined directly by a LEICA TCS Laser scan microscope equipped with 488-nm argon-ion laser and with 543 nm helium–neon ion laser. Optical sections were collected at different levels perpendicular to the optical axis. Photomultiplier gain and laser power were identical within each experiment.

Cytotoxicity

NIH 3T3 cells were incubated for 60 min at 37 °C in DMEM, in the absence or presence of each peptide. The activity of the cytosolic enzyme lactate dehydrogenase (LDH) was measured in cell-free media and cell lysates using the CytoTox 96 non-radioactive cytotoxicity assay (Promega). LDH activity in the culture media was expressed as percent of total cellular LDH activity. Permeabilization to PI was evaluated in peptide-treated cells after staining with 100 μM PI, 1 mg/ml RNase in PBS for 20 min. Cells were mounted on glass slides and examined using a fluorescence microscope (Nikon) equipped with TRITC filters. The number of PI-positive nuclei was expressed as percentage of total nuclei.

Cell Proliferation Assays

NIH 3T3 cells (1×10^4 cells/cm²) were seeded on coverslips in 35-mm-diameter dishes or in 96-well plates and

incubated at 37 °C in complete DMEM. After 24 h, the cell culture medium was replaced with DMEM containing 0.5% FCS and the incubation was prolonged for 48 h. After this time, addition of 50 μM bromodeoxyuridine (BrdU) for 60 min resulted in less than 5% of BrdU-positive nuclei. Growth-arrested cells were incubated in DMEM containing 2% FCS and 50 μM BrdU, in the absence or presence of the selected peptide. After 18 h, cells were fixed with 4% PFA in PBS and processed for immunofluorescence as described [10]. DNA synthesis was assessed by counts of BrdU-positive cell nuclei over total cell nuclei after BrdU incorporation. Alternatively, growth-arrested cells were incubated in DMEM containing 2% FCS, in the absence or presence of the selected peptide. After 24 h incubation, 0.5 mg/ml methyl thiazole tetrazolium (MTT) was added to the culture media and cells were incubated for an additional 3 h. Cells were then lysed with isopropanol, and the absorbance at 570 nm was recorded. Cell proliferation in the presence of each peptide was expressed as percentage of the control value, corresponding to untreated cells. Statistical differences among groups of data were analyzed by one-way ANOVA followed by Bonferroni post-test, using GraphPad Prism version 5.0 (GraphPad Software, Inc.). In all comparisons, $P < 0.05$ was considered significant.

Results

The peptides investigated in this work are listed in Table 1, and their antimicrobial activities are shown in Table 2. The helical cathelicidin BMAP-28 and the proline-rich Bac7(1–35), in particular, were tested on a range of bacteria and a number of strains for each micro-organism. For all the peptides, the activity of the all-*D* enantiomer was also tested against representative strains.

The mode-of-action of the different peptides was probed by monitoring the uptake of PI using flow cytometry, a standard method for detecting if they damage bacterial or eukaryotic cells. Treatment of representative Gram-positive or Gram-negative bacteria at 1 μM BMAP-28 resulted in marked permeabilization (Table 2), with effectively all cells showing PI positive already after 15 min incubation, and the all-*D* enantiomer behaved in an entirely similar manner. Conversely, treatment of bacteria with the proline-rich Bac5 and Bac7(1–35) resulted in little permeabilization even though the 1 μM concentration is at the high end of the measured MIC values for *E. coli*.

The mode of action of the peptides was further studied by making use of fluorescently labeled peptides [5]. When *E. coli* cells were incubated with BODIPY-labeled Bac7(1–35), flow cytometric analysis showed a considerable increase in cell fluorescence with respect to an untreated

Table 1 Primary structures and properties of the cathelicidin AMPs used in this study

Peptide	Sequence				MW [Da]	Charge	AA	\bar{H}^a
	10	20	30	40				
Bac5	RFRPPPIRRPPPIRPPFYPPFRPPPIRPPPIFPPPIRPPFRPPLGPPF-NH ₂				5148.3	10	43	0.44
Bac7(1-35)	RRIRPRPRLPRPRRPLPFPRGPRPIRPLPFP-OH				4207.2	11	35	-1.4
BMAP-28	GGLRSLGRKILRAWKKYGPIIVPIIRI-NH ₂				3073.9	8	27	0.46

^a per residue average hydrophobicity calculated using the CCS hydrophobicity index scale (<http://www.bbcm.univ.trieste.it/~tossi/HydroCalc/HydroMCalc.html>). In this scale, the more positive the index, the more hydrophobic the residue is. Polar, hydrophilic residues have negative indices

Table 2 Antimicrobial and permeabilizing activities of the cathelicidin AMPs and analogs used in this study

Peptides	BMAP-28	D-BMAP-28	BAC5	D-BAC5	BAC7(1–35)	D-BAC7(1–35)
Antimicrobial activity (MIC, μ M) ^a						
<i>E. coli</i>	1–8 (9) ^b	1–2 ^c	0.5 ^c	8 ^c	0.125–1 (10)	16 ^c
<i>P. aeruginosa</i>	2–8 (10)	–	8 ^c	–	0.5–32 (20)	>64 ^c
<i>K. pneumoniae</i>	1–8 (12)	–	1 ^c	–	0.25–1 (13)	–
<i>S. enteritidis</i>	4–8 (10)	–	1 ^c	–	0.125–0.5 (10)	>64 ^c
<i>S. aureus</i>	1–4 (10)	2–4 ^c	>64 ^c	>128 ^c	> 128 ^c	>128 ^c
<i>C. albicans</i>	2–16 (24)	–	–	–	>64 (6)	–
<i>C. neoformans</i>	0.5–4 (17)	–	–	–	1–32 (13)	–
Bacterial cell permeabilization (percentage PI positive cells) ^d						
<i>E. coli</i> ATCC25922	95%	85%	5%	10%	<5%	5%
<i>S. aureus</i> ATCC25923	97%	97%	<5%	<5%	<5%	<5%

^a Data are a compendium of unpublished and previously published results [4, 24, 27, 29]. MIC values were determined in Mueller–Hinton broth for bacteria (5×10^5 CFU/ml), and RPMI-1640 medium for yeast (5×10^4 CFU/ml) using micro-organisms at logarithmic phase, and are the mean of at least three independent experiments performed in duplicate

^b Numbers in parenthesis indicate the number of different strains on which MIC determinations were carried out; the reported MIC values indicate the range

^c Data refer to the *E. coli* ATCC 25922, *S. aureus* ATCC 25923, *S. enteritidis* ATCC 14028, *K. pneumoniae* ATCC 13883 and *P. aeruginosa* ATCC 27853 strains

^d Percentage PI positive cells, calculated from flow-cytometric data after 15 min incubation with 1 μ M peptide

population (Fig. 1a, MFI shifts from 10 to 230). Extensive washing followed by exposure to the cell impermeant quencher TB would significantly reduce fluorescence deriving from external or surface-bound fluorophore, but would not affect internalized fluorophore in the absence of membrane lesions. As reported in Fig. 1, washing and treatment with TB hardly affects fluorescence of *E. coli* cells exposed to labeled Bac7(1–35) (MFI = 220, < 5% quenching), confirming it has virtually all internalized into cells without causing membrane damage. The cellular internalization of Bac7(1–35) on the same cell preparation was confirmed by confocal microscopy (Fig. 2). Treatment of *E. coli* cells with 0.25 μ M BODIPY-labeled peptide for 10 min resulted in a homogeneous cytoplasmic staining, as

can be seen in a representative section from the middle of a bacterial cell.

Exposure to fluorescein-labeled BMAP-28 also increased the fluorescence of *E. coli* (Fig. 1b, MFI shifts from 25 to 190), but in this case, both washing and quenching with TB resulted in a significant reduction of fluorescence (MFI = 50, 74% quenching), indicating that it remains accessible to the quencher.

The differential capacities of the peptides to interact with animal cells was probed by incubating 1.5 μ M fluorescein-conjugated BMAP-28, Bac5 or Bac7(1–35) with NIH 3T3 in the presence of 2% heat-inactivated FCS. Confocal microscopic analysis of these cells revealed that at these concentrations all peptides rapidly translocated

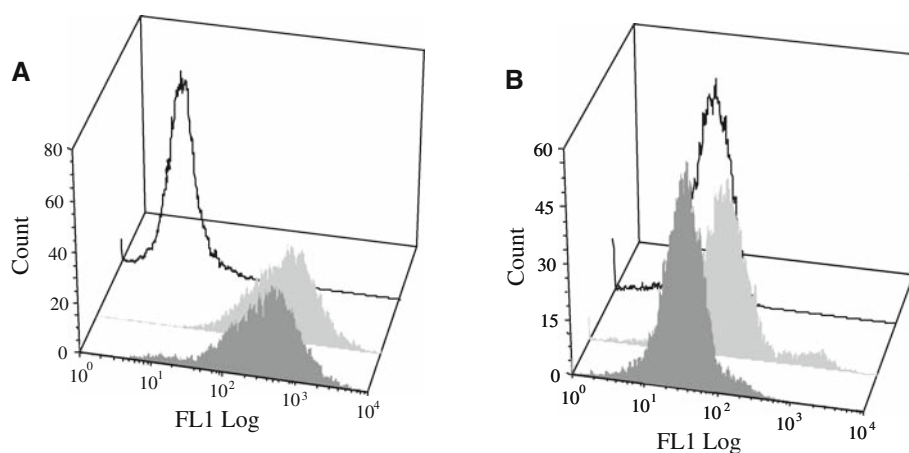


Fig. 1 Internalization of cathelicidin peptides into bacterial cells. Effect of Trypan Blue on the fluorescence of *E. coli* HB101 cells exposed to Bac7(1–35)-BODIPY (BP) (a) or BMAP-28-Fluorescein (FL) (b). *E. coli* cells (1×10^6 CFU/ml) were incubated with

0.25 μ M Bac7(1–35)-BY or BMAP-28-FL for 10 min, washed with buffered high-salt solution, and then analyzed by flow cytometry with (dark gray) or without (light gray) incubation with 1 mg/ml of TB for 10 min. Untreated cells are shown by the empty histogram

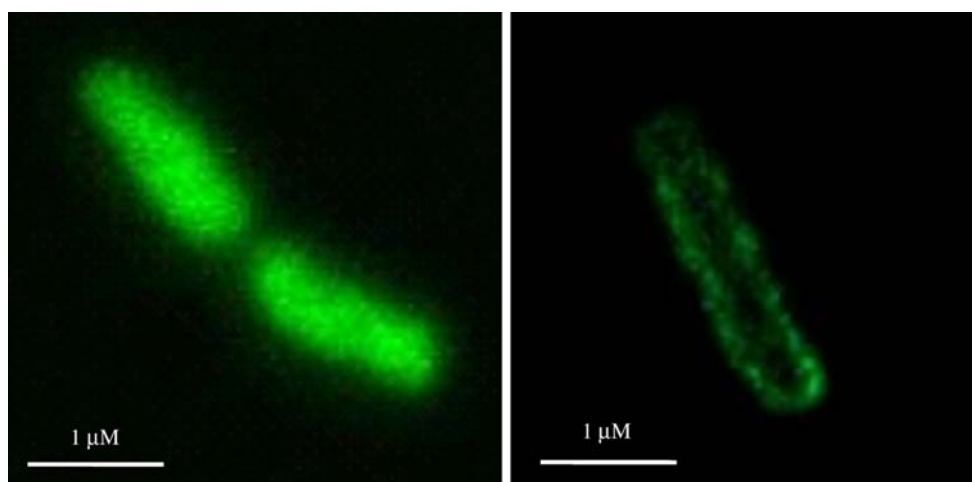


Fig. 2 Bac7(1–35) internalization into Gram-negative bacterial cells. Confocal microscopic images of *E. coli* HB101 cells upon incubation for 10 min with 0.25 μ M Bac7(1–35)-BY (left) or an anti-LPS, FITC-labeled antibody (right). All images are representative sections from

the middle of the bacterial cell. Over 95% of examined cells displayed microscopic images equivalent to those shown in the above representative images

through the lipid bilayer without cell permeabilization and were found associated with intracellular structures after 15 min of incubation, as shown in Fig. 3. The α -helical peptide BMAP-28 showed a punctate cytoplasmic distribution of the staining (Fig. 3a, d) with partial co-localization with the mitochondrial cytochrome oxidase (Fig. 3e, f). The proline-rich Bac5 (Fig. 3b) and Bac7(1–35) (Fig. 3c) were instead mostly associated with nuclear (Bac 5) or nucleolar [Bac 7(1–35)] structures.

To investigate-growth promoting activities of the peptides under study, NIH 3T3 fibroblasts synchronized in G_0 by growth-arrest, were incubated in DMEM containing 2% FCS in the absence (control) or presence of increasing concentrations of each peptide. Bac5 induced proliferation

at concentrations above 10 μ M, as determined using the BrdU incorporation assay, with an efficiency at 25 μ M slightly lower than that of 10% FCS-containing medium (Fig. 4a). The MTT assay confirmed proliferation promoting activity for both Bac5 and Bac7(1–35), whereas BMAP-28 was ineffective at up to 3 μ M (Fig. 4b) and caused a dose-dependent decrease in the cell numbers at higher doses (data not shown) suggesting that at these concentrations it was toxic to the cells.

The cytotoxic potential of this peptide in eukaryotic cells was thus further investigated using both the PI permeabilization and LDH release assays. In the former assay, the formation of even small lesions to the cytoplasmic membrane can allow the rapid access of PI (MW = 668)

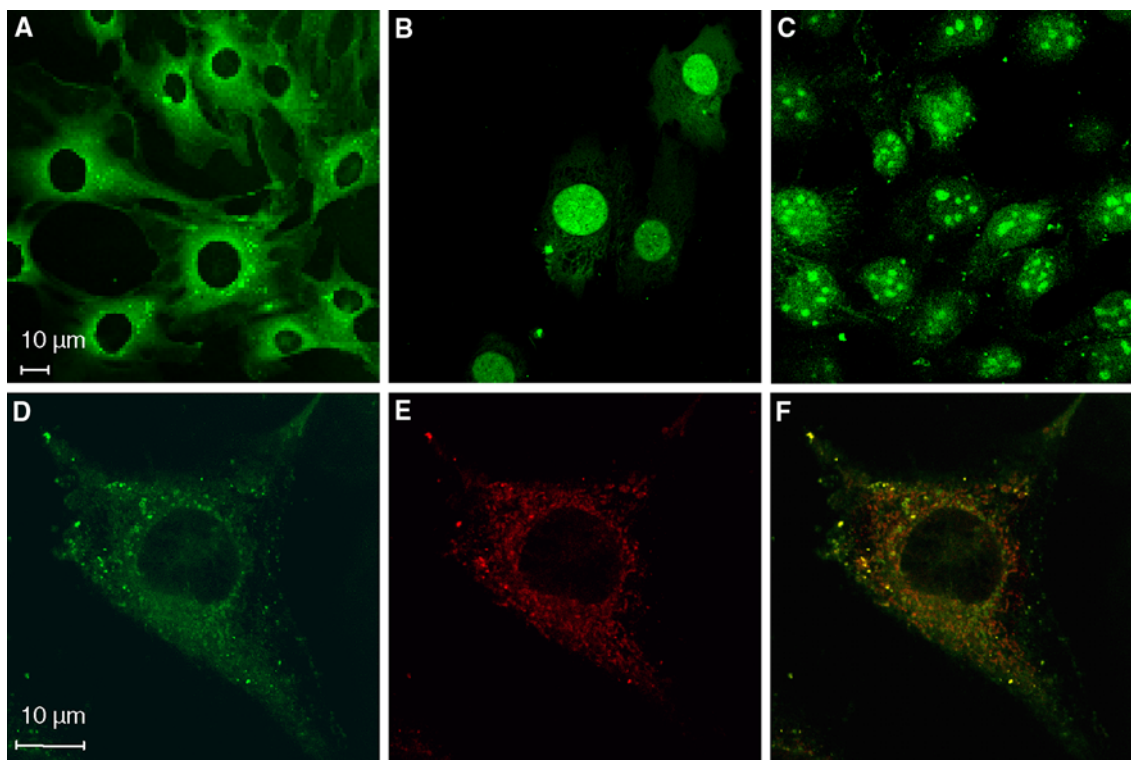


Fig. 3 Internalization and localization of cathelicidin peptides in NIH 3T3 cells. Cells were treated for 15 min with 1.5 μM fluorescein-conjugated BMAP-28 (a), Bac5 (b) or Bac7(1–35) (c) and observed unfixed by confocal laser microscopy. Confocal analysis of

cells exposed to 1.5 μM fluorescein-conjugated BMAP-28 for 15 min (d), fixed with paraformaldehyde and incubated with antibodies to cytochrome oxidase (e) (TRITC secondary antibodies), and superimposition of the two (f)

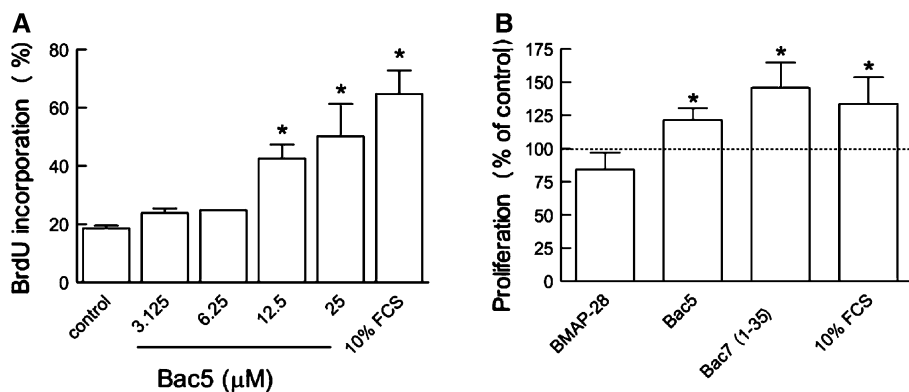


Fig. 4 Effect of cathelicidin peptides on proliferation of NIH 3T3 cells. Growth-arrested NIH 3T3 cells were incubated in DMEM containing 2% FCS in the absence (control) or presence of increasing concentrations of Bac5 (a) or in the presence of BMAP-28 (3 μM), Bac5 (25 μM) or Bac7(1–35) (25 μM) (b). As a cell growth control, cells were incubated with 10% FCS-containing medium. The induction of DNA synthesis was assessed after 18 h incubation in

the presence of 50 μM BrdU (a) and expressed as the ratio between BrdU-positive nuclei and total nuclei. The number of cells after 24 h incubation was assessed by MTT assay (b) and was expressed as percent of cells incubated in the absence of each peptide. The mean of at least three independent experiments and the SD are reported. (* $P < 0.05$ as assessed by one-way analysis of variance and Bonferroni post-test)

and the effect can be assessed already after quite short exposure to the peptides, whereas in the latter assay, larger lesions are required for release of the enzyme from the cytoplasm. BMAP-28 showed some toxicity against NIH 3T3 fibroblasts already at 3 μM, as determined by both uptake of PI and LDH

release (Fig. 5a), in a serum-dependent manner (Fig. 5b). The corresponding all-*D* enantiomer was slightly more cytotoxic (data not shown). On the contrary, exposure to up to 50 μM Bac5 or Bac7(1–35) did not cause significant membrane permeabilization (Fig. 5a).

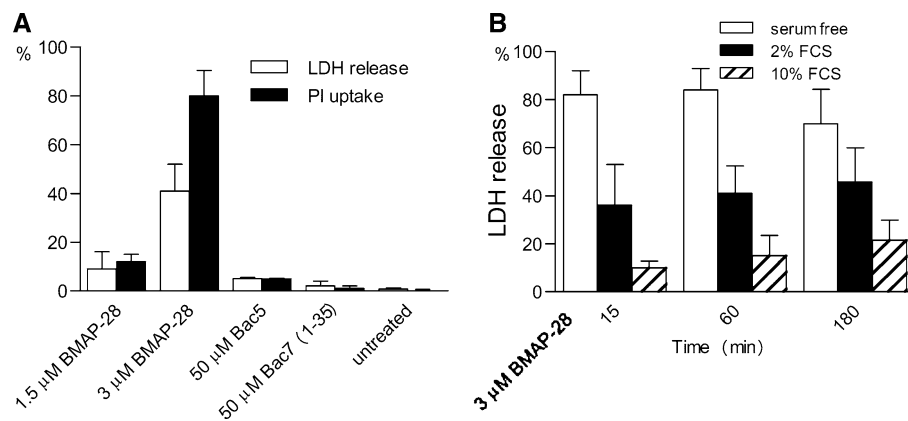


Fig. 5 Cytotoxic activity of cathelicidin peptides. Effect of BMAP-28, Bac5 and Bac7(1–35) on the integrity of plasma membrane of NIH 3T3 cells. **(a)** LDH activity was measured in the cell supernatant after 1 h incubation in DMEM, 2% FCS, in the absence or presence of the indicated concentrations of BMAP-28, Bac5 or Bac7(1–35) and expressed as percent of total cellular LDH activity. Positivity to

propidium iodide was evaluated by fluorescence microscopy analysis of NIH 3T3 cells treated with the same peptide concentrations and expressed as percent of total cell number after Hoechst staining. **(b)** LDH activity measured after 15, 60 and 180 min incubation of NIH 3T3 cells with 3 μ M BMAP-28 in DMEM containing increasing amounts of FCS

Discussion

The bovine cathelicidin Bac5 is a proline- and arginine-rich cathelicidin of 43 residues with an amidated C-terminus and charge 10 + (Table 1). Bac7 is longer (60 residues), but it was found that the 1–35 N-terminal fragment, Bac7(1–35), had an equivalent activity to the parent peptide on susceptible Gram-negative bacteria [3]. This peptide is also highly cationic (11 +) and more hydrophilic than Bac5. BMAP-28 (charge 8 +) has a comparable hydrophobicity to Bac5 and undergoes a transition from an unstructured to an α -helical conformation in the presence of a membrane-like environment [22], unlike the two proline-rich peptides, which likely remain in an extended conformation in the presence of bacterial membranes [1, 23].

These peptides differed significantly in the range and potency of their antimicrobial activity. BMAP-28 showed a broad-spectrum in vitro activity covering Gram-positive and Gram-negative bacteria as well as yeasts, although a significant variation in potency was observed with respect to different strains and isolates of the same micro-organism. Furthermore, assays with selected Gram-positive and -negative bacteria indicated that the all-*D* enantiomer displayed a similar activity, in accordance with a proposed membranolytic mechanism initiating with the interaction of the amphipathic helix with the membrane surface. The helix sense makes little difference to the formation of a hydrophobic face on the helix, a structural feature required for subsequent insertion into the lipid bilayer. The proline-rich Bac5 and Bac7 were particularly active against Gram-negative bacteria, but generally not against Gram-positive ones. They displayed a potent activity against all *E. coli*, *K. pneumoniae* and *S. enteritidis* strains tested, whereas some *P. aeruginosa* isolates were quite resistant to Bac7

(1–35). Significantly, the available genomes of this Gram-negative bacteria lack the gene for the SbmA membrane transport system (see the next paragraph). Furthermore, although Bac7(1–35) was inactive against all tested *C. albicans* yeast isolates, several *C. neoformans* yeast isolates showed a considerable susceptibility to this peptide, although the mechanism of cidal action remains unknown. In the case of the proline-rich AMPs, the all-*D* enantiomers showed a significant loss in potency against selected susceptible Gram-negative isolates. This is in agreement with a proposed mechanism partially depending on active transport through the cytoplasmic membrane involving the SbmA protein, which presumably requires a stereoselective recognition [13, 19]. There is also evidence that the subsequent interaction with internal targets, leading to cellular inactivation, also requires a stereo-selective binding [20].

The different modes-of-action of BMAP-28 (membranolytic) and proline-rich Bac5 or Bac7(1–35) (non-lytic), was confirmed by uptake of PI (Table 2). Results clearly showed that BMAP-28 acted via a membranolytic mechanism at low micromolar concentrations, whereas the proline-rich peptides were non-lytic at concentrations lethal to susceptible bacteria. Furthermore, by using fluorescently labeled peptides and an extracellular, impermeant quencher (Fig. 1), it was possible to determine that BMAP-28 either remained bound to the membrane surface, or, if internalized, remained accessible due the presence of lesions that allowed access to it by the quencher. The labelled proline-rich Bac5 and Bac7(1–35) instead internalized into susceptible bacterial cells without cell lysis, and were thus protected from the quencher. Cellular internalization was confirmed by confocal microscopy of *E. coli* cells treated with fluorescently labeled Bac7(1–35) (Fig. 2), which resulted in a homogeneous cytoplasmic staining.

The different mechanisms of antibacterial action of the helical BMAP-28 and the proline-rich Bac5 and Bac7(1–35) derive from distinctly different structures, orienting the peptides toward different targets. To investigate whether these structural features also affect interactions with host cells, we next probed for a differential ability of fluorescein-conjugated BMAP-28, Bac5 or Bac7(1–35) to interact with and become internalized into fibroblasts, as a model mammalian cell. Confocal microscopic analysis of NIH 3T3 cells revealed that at low micromolar concentrations all peptides translocated rapidly through the lipid bilayer without permeabilization (Fig. 3) and associated with intracellular structures. BMAP-28 associated with mitochondria, while Bac5 and Bac7(1–35) with nuclear or nucleolar structures.

The interaction of BMAP-28, Bac5 or Bac7(1–35) with host cells might result in the induction of cellular effects. In this respect, it has been reported that some cathelicidins have the ability to induce cellular proliferation, a process which could be associated with wound healing [32]. We found that the proline-rich peptides did efficiently induce proliferation at concentrations above 10 μM (Fig. 4), whereas BMAP-28 was ineffective up to 3 μM , and in fact was likely toxic to these cells at higher doses. The cytotoxic potential of this peptide on eukaryotic cells was thus further investigated using membrane permeabilization assays, and in fact showed some toxicity against NIH 3T3 fibroblasts already at 3 μM (Fig. 5), while exposure to Bac5 or Bac7(1–35) up to 50 μM did not cause significant membrane permeabilization. It was interesting that the all-*D* enantiomer of BMAP-28 was slightly more cytotoxic than the all-*L* peptide, a feature observed also with model helical AMPs [14]. This may be because it is less susceptible to interactions with the medium components, outer wall components or proteolytic enzymes, which may have a protective effect (see the next paragraph).

It is not surprising, considering that the proline-rich Bac5 and Bac7(1–35) do not display a membranolytic mechanism against bacteria, that they should also not display such an activity on eukaryotic cells. On the other hand, it would appear that the cell membrane is a primary target of the α -helical BMAP-28 in both bacterial and eukaryotic cells. In a prior study, we have shown that in the presence of toxic concentrations of BMAP-28, its membrane perturbing activity is associated to apoptotic cell death through opening of the mitochondrial membrane transition pore [18]. It should be considered, however, that, owing to the ability of hydrophobic serum components to bind cationic and amphipathic peptides [37], the cell permeabilization by BMAP-28 is dose-dependently inhibited by serum, as shown in Fig 5b. The sequestering effect of serum would decrease the effective concentration of BMAP-28 under physiological conditions and may thus

represent a means to protect the host cells from untoward peptide-mediated toxicity [6]. It is interesting in this respect that, although in the present study BMAP-28 was incapable to induce cell proliferation at non-toxic peptide concentrations, we have observed in another study that it induces cytokine expression in epithelial cells at similarly low peptide concentrations [28], suggesting it may contribute through this mechanism to the activation of innate immune responses in addition to exerting direct antimicrobial activities.

Conclusions

In this work, we have compared the antimicrobial activity, the host-cell interaction properties and the cytotoxicity of an α -helical and two proline-rich bovine cathelicidins. These show distinctly different spectra and modes of antimicrobial action. The former peptide has a principally membranolytic activity which correlates with a broader spectrum, while the latter peptides act via a non-lytic internalization mechanism on susceptible Gram-negative species, resulting in a narrower spectrum of activity. These functional features may also reflect on their effects on host cells, with the proline-rich peptides displaying non-detectable cytotoxicity and an efficient internalization, followed by nuclear localization, while the helical BMAP-28 shows graded effects on host cells, ranging from induction of cytokine release at non-lytic concentrations, which may help activate innate immune responses, to pro-apoptotic effects at near-lytic concentrations, that may provide cells with the possibility to contain potential necrotic effects.

In this respect, it is interesting to consider recent reports that have indicated an increased presence of bovine cathelicidins in conceptus fluid in response to immuno-inflammatory episodes [16], as well as in bovine milk during experimentally induced mastitis [7]. We have recently found that these cathelicidins (helical, proline-rich, as well as indolicidin), may have a collaborative antimicrobial activity in milk or whey [28]. All these considerations indicate that the different types of cathelicidins expressed in bovines may contribute to the integrated response of the host to infection, with distinct but complementary effects, and underline their potential for the development of novel therapeutic agents or protocols for veterinary use.

References

1. Abiraj K, Prasad HS, Gowda AS, Gowda DC (2004) Design, synthesis and antibacterial activity studies of model peptides of proline/arginine-rich region in bacteriocin7. *Protein Pept Lett* 11:291–300

2. Benincasa M, Skerlavaj B, Gennaro R, Pellegrini A, Zanetti M (2003) In vitro and in vivo antimicrobial activity of two alpha-helical cathelicidin peptides and of their synthetic analogs. *Peptides* 24(11):1723–1731
3. Benincasa M, Scocchi M, Podda E, Skerlavaj B, Dolzani L, Gennaro R (2004) Antimicrobial activity of Bac7 fragments against drug-resistant clinical isolates. *Peptides* 25:2055–2061
4. Benincasa M, Scocchi M, Pacor S, Tossi A, Nobili D, Basaglia G, Busetti M, Gennaro R (2006) Fungicidal activity of five cathelicidin peptides against clinically isolated yeasts. *J Antimicrob Chemother* 58:950–959
5. Benincasa M, Pacor S, Gennaro R, Scocchi M (2009) Rapid and reliable detection of antimicrobial peptide penetration into gram-negative bacteria based on fluorescence quenching. *Antimicrob Agents Chemother* 53:3501–3504
6. Björstad A, Askarieh G, Brown KL, Christenson K, Forsman H, Onnheim K, Li HN, Teneberg S, Maier O, Hoekstra D, Dahlgren C, Davidson DJ, Bylund J (2009) The host defense peptide LL-37 selectively permeabilizes apoptotic leukocytes. *Antimicrob Agents Chemother* 53(3):1027–1038
7. Boehmer JL, Bannerman DD, Shefcheckm K, Wardm JL (2009) Proteomic analysis of differentially expressed proteins in bovine milk during experimentally induced *Escherichia coli* mastitis. *J Dairy Sci* 91(11):4206–4218
8. Bowdish DM, Davidson DJ, Scott MG, Hancock RE (2005) Immunomodulatory activities of small host defense peptides. *Antimicrob Agents Chemother* 49:1727–1732
9. Bowdish DM, Davidson DJ, Hancock RE (2006) Immunomodulatory properties of defensins and cathelicidins. *Curr Top Microbiol Immunol* 306:27–66
10. Goruppi S, Ruaro E, Varnum B, Schneider C (1997) Requirement of phosphatidylinositol 3-kinase-dependent pathway and Src for Gas6-Axl mitogenic and survival activities in NIH 3T3 fibroblasts. *Mol Cell Biol* 17:4442–4453
11. Hancock RE, Sahl HG (2006) Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nat Biotechnol* 24:1551–1557
12. Lai Y, Gallo RL (2009) AMPed up immunity: how antimicrobial peptides have multiple roles in immune defense. *Trends Immunol* 30:131–141
13. Mattiuzzo M, Bandiera A, Gennaro R, Benincasa M, Pacor S, Antcheva N, Scocchi M (2007) Role of the *Escherichia coli* SbmA in the antimicrobial activity of proline-rich peptides. *Mol Microbiol* 66(1):151–163
14. Pacor S, Giangaspero A, Bacac M, Sava G, Tossi A (2002) Analysis of the cytotoxicity of synthetic antimicrobial peptides on mouse leucocytes: implications for systemic use. *J Antimicrob Chemother* 50:339–348
15. Rehaume LM, Hancock RE (2008) Neutrophil-derived defensins as modulators of innate immune function. *Crit Rev Immunol* 28:185–200
16. Riding GA, Hill JR, Jones A, Holland MK, Josh PF, Lehnert SA (2008) Differential proteomic analysis of bovine conceptus fluid proteins in pregnancies generated by assisted reproductive technologies. *Proteomics* 8(14):2967–2982
17. Risso A, Zanetti M, Gennaro R (1998) Cytotoxicity and apoptosis mediated by two peptides of innate immunity. *Cell Immunol* 189:107–115
18. Risso A, Braidot E, Sordano MC, Vianello A, Macrì F, Skerlavaj B, Zanetti M, Gennaro R, Bernardi P (2002) BMAP-28, an antibiotic peptide of innate immunity, induces cell death through opening of the mitochondrial permeability transition pore. *Mol Cell Biol* 22:1926–1935
19. Scocchi M, Mattiuzzo M, Benincasa M, Antcheva N, Tossi A, Gennaro R (2008) Investigating the mode of action of proline-rich antimicrobial peptides using a genetic approach: a tool to identify new bacterial targets amenable to the design of novel antibiotics. *Methods Mol Biol* 494:161–176
20. Scocchi M, Lüthy C, Decarli P, Mignogna G, Christen P, Gennaro R (2009) The proline-rich bactenecin Bac7 binds to and inhibits the molecular chaperone DnaK. *Int J Pep Res Ther* 15:147–156
21. Selsted ME, Ouellette AJ (2005) Mammalian defensins in the antimicrobial immune response. *Nat Immunol* 6:551–557
22. Skerlavaj B, Gennaro R, Bagella L, Merluzzi L, Risso A, Zanetti M (1996) Biological characterization of two novel cathelicidin-derived peptides and identification of structural requirements for their antimicrobial and cell lytic activities. *J Biol Chem* 271(45):28375–28381
23. Tokunaga Y, Niidome T, Hatakeyama T, Aoyagi H (2001) Antibacterial activity of bactenecin 5 fragments and their interaction with phospholipid membranes. *J Pept Sci* 7:297–304
24. Tomasinsig L, Zanetti M (2005) The cathelicidins—structure, function and evolution. *Curr Protein Pept Sci* 6:23–34
25. Tomasinsig L, Scocchi M, Di Loreto C, Artico D, Zanetti M (2002) Inducible expression of an antimicrobial peptide of the innate immunity in polymorphonuclear leukocytes. *J Leukoc Biol* 72:1003–1010
26. Tomasinsig L, Skerlavaj B, Papo N, Giabbai B, Shai Y, Zanetti M (2006) Mechanistic and functional studies of the interaction of a proline-rich antimicrobial peptide with mammalian cells. *J Biol Chem* 281(1):383–391
27. Tomasinsig L, Pizzirani C, Skerlavaj B, Pellegatti P, Gulinelli S, Tossi A, Di Virgilio F, Zanetti M (2008) The human cathelicidin LL-37 modulates the activities of the P2X7 receptor in a structure-dependent manner. *J Biol Chem* 283(45):30471–30481
28. Tomasinsig L, De Conti G, Skerlavaj B, Piccinini R, Mazzilli M, D'Este F, Tossi A, Zanetti M (2010) Broad spectrum activity against bacterial mastitis pathogens and activation of mammary epithelial cells support a protective role of neutrophil cathelicidins in bovine mastitis. *Infect Immun* (in press)
29. Tossi A, Sandri L (2002) Molecular diversity in gene-encoded, cationic antimicrobial polypeptides. *Curr Pharm Des* 8:743–761
30. Waddell WJ (1956) A simple ultraviolet spectrophotometric method for the determination of protein. *J Lab Clin Med* 48: 311–334
31. Yang D, de la Rosa G, Tewary P, Oppenheim JJ (2009) Alarmins link neutrophils and dendritic cells. *Trends Immunol* (Epub ahead of print)
32. Zanetti M (2004) Cathelicidins, multifunctional peptides of the innate immunity. *J Leukoc Biol* 75:39–48
33. Zanetti M (2005) The role of cathelicidins in the innate host defenses of mammals. *Curr Issues Mol Biol* 7(2):179–196
34. Zanetti M, Gennaro R, Romeo D (1995) Cathelicidins: a novel protein family with a common proregion and a variable C-terminal antimicrobial domain. *FEBS Lett* 374(1):1–5
35. Zasloff M (2002) Antimicrobial peptides of multicellular organisms. *Nature* 415(6870):389–395
36. Zelezetsky I, Pontillo A, Puzzi L, Antcheva N, Segat L, Pacor S, Crovella S, Tossi A (2006) Evolution of the primate cathelicidin. Correlation between structural variations and antimicrobial activity. *J Biol Chem* 281:19861–19871
37. Zhang Z, Cherryholmes G, Shively JE (2008) Neutrophil secondary necrosis is induced by LL-37 derived from cathelicidin. *J Leukoc Biol* 84(3):780–788